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Infect. Immun 1989, 57(8), 2553-2558
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(54) **Cysteine-free streptolysin O**

(57) A derivative of the thiol-activated toxin streptolysin O, which contains no cysteine amino acids but retains cytolytic activity which can be neutralised by anti-SLO antibodies in a sample.

The claims were filed later than the filing date within the period prescribed by Rule 25(1) of the Patents Rules 1982.

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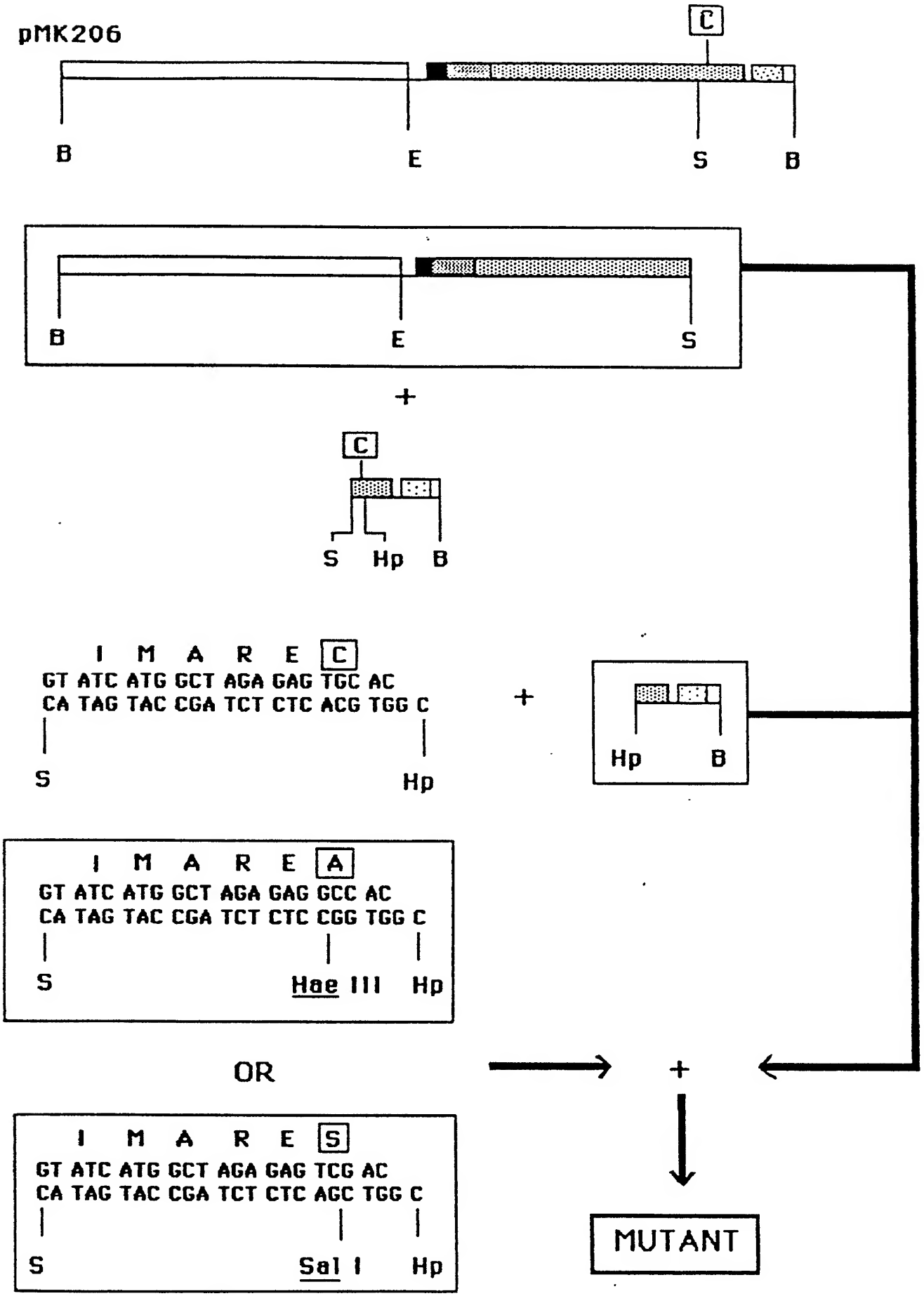
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CA TAG TAC CGA TCT CTC ACG TGG C
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B
I M A R E A
GT ATC ATG GCT AGA GAG GCC AC
CA TAG TAC CGA TCT CTC CGG TGG C
S
Hae III
Hp
OR
I M A R E S
GT ATC ATG GCT AGA GAG TCG AC
CA TAG TAC CGA TCT CTC AGC TGG C
S
Sal I
Hp
+
MUTANT

FIGURE 1

Figure —

A. NATIVE SLO SEQUENCE

SLO.DNA

```
                                                                    60
TTATTAGCAAGCTTGCCATTTTATTTAAACCGTCAAAGCATACTAGCTAATATAACCAAA

                                                                   120
GCGTTAAAAGATGCGCATTATTAGAGAGGCTATGGCACATTACAAATTAGGAGAATTTGC

                                                                   180
TCACTATCAAGATACTATGCTTGATATGGTCGAAAGAACAATAGAAACATTTTAGAATGA

                                                                   240
TAAAAAGGTATGAAGGACATGTCTAATAAAAAAACATTTAAAAAATACAGTCGCGTCGCT
                      M  S  N  K  K  T  F  K  K  Y  S  R  V  A

                                                                   300
GGGCTACTGACGGCAGCTCTTATCATTGGTAACCTTGTTACTGCTAATGCTGAATCGAAC
 G  L  L  T  A  A  L  I  I  G  N  L  V  T  A  N  A  E  S  N

                                                                   360
AAACAAAACACTGCTAGTACAGAAACCACAACGACAAATGAGCAACCAAAGCCAGAAAGT
 K  Q  N  T  A  S  T  E  T  T  T  T  N  E  Q  P  K  P  E  S

                                                                   420
AGTGAGCTAACTACTGAAAAAGCAGGTCAGAAAACGGATGATATGCTTAACTCTAACGAT
 S  E  L  T  T  E  K  A  G  Q  K  T  D  D  M  L  N  S  N  D

                                                                   480
ATGATTAAGCTTGCTCCCAAAGAAATGCCACTAGAATCTGCAGAAAAAGAAGAAAAAAAG
 M  I  K  L  A  P  K  E  M  P  L  E  S  A  E  K  E  E  K  K

                                                                   540
TCAGAAGACAAAAAAAAGAGCGAAGAAGATCACACTGAAGAAATCAATGACAAGATTTAT
 S  E  D  K  K  K  S  E  E  D  H  T  E  E  I  N  D  K  I  Y

                                                                   600
TCACTAAATTATAATGAGCTTGAAGTACTTGCTAAAAATGGTGAAACCATTGAAAATTTT
 S  L  N  Y  N  E  L  E  V  L  A  K  N  G  E  T  I  E  N  F

                                                                   660
GTTCCTAAAGAAGGCGTTAAGAAAGCTGATAAATTTATTGTCATTGAAAGAAAGAAAAAA
 V  P  K  E  G  V  K  K  A  D  K  F  I  V  I  E  R  K  K  K

                                                                   720
AATATCAACACTACACCAGTCGATATTTCCATCATTGACTCTGTCACTGATAGGACCTAT
 N  I  N  T  T  P  V  D  I  S  I  I  D  S  V  T  D  R  T  Y

                                                                   780
CCAGCAGCCCTTCAGCTGGCTAATAAAGGTTTTACCGAAAACAAACCAGACGCGGTAGTC
 P  A  A  L  Q  L  A  N  K  G  F  T  E  N  K  P  D  A  V  V

                                                                   840
ACCAAGCGAAACCCACAAAAAATCCATATTGATTTACCAGGTATGGGAGACAAAGCAACG
 T  K  R  N  P  Q  K  I  H  I  D  L  P  G  M  G  D  K  A  T

                                                                   900
GTTGAGGTCAATGACCCTACCTATGCCAATGTTTCAACAGCTATTGATAATCTTGTTAAC
 V  E  V  N  D  P  T  Y  A  N  V  S  T  A  I  D  N  L  V  N
```

```
                                                              960
CA TGGCATGATAATTATTCTGGTGGTAATACGCTTCCTGCCAGAACACAATATACTGAA
 L  W  H  D  N  Y  S  G  G  N  T  L  P  A  R  T  Q  Y  T  E

                                                             1020
TCAATGGTATATTCTAAGTCACAGATTGAAGCAGCTCTAAATGTTAATAGCAAAATCTTA
 S  M  V  Y  S  K  S  Q  I  E  A  A  L  N  V  N  S  K  I  L

                                                             1080
GATGGTACTTTAGGCATTGATTTCAAGTCGATTTCAAAAGGTGAAAAGAAGGTGATGATT
 D  G  T  L  G  I  D  F  K  S  I  S  K  G  E  K  K  V  M  I

                                                             1140
GCAGCATACAAGCAAATTTTTTACACCGTATCAGCAAACCTTCCTAATAATCCTGCGGAT
 A  A  Y  K  Q  I  F  Y  T  V  S  A  N  L  P  N  N  P  A  D

                                                             1200
GTGTTTGATAAATCAGTGACCTTTAAAGAGTTGCAACGAAAAGGTGTCAGCAATGAAGCT
 V  F  D  K  S  V  T  F  K  E  L  Q  R  K  G  V  S  N  E  A

                                                             1260
CCGCCACTCTTTGTGAGTAACGTAGCCTATGGTCGAACTGTTTTTGTCAAACTAGAAACA
 P  P  L  F  V  S  N  V  A  Y  G  R  T  V  F  V  K  L  E  T

                                                             1320
AGTTCTAAAAGTAATGATGTTGAAGCGGCCTTTAGTGCAGCTCTAAAAGGAACAGATGTT
 S  S  K  S  N  D  V  E  A  A  F  S  A  A  L  K  G  T  D  V

                                                             1380
AAAACTAATGGAAAATACTCTGATATCTTAGAAAATAGCTCATTTACAGCTGTCGTTTTA
 K  T  N  G  K  Y  S  D  I  L  E  N  S  S  F  T  A  V  V  L

                                                             1440
GGAGGAGATGCTGCAGAGCACAATAAGGTAGTCACAAAAGACTTTGATGTTATTAGAAAC
 G  G  D  A  A  E  H  N  K  V  V  T  K  D  F  D  V  I  R  N

                                                             1500
GTTATCAAAGACAATGCTACCTTCAGTAGAAAAAACCCAGCTTATCCTATTTCATACACC
 V  I  K  D  N  A  T  F  S  R  K  N  P  A  Y  P  I  S  Y  T

                                                             1560
AGTGTTTTCCTTAAAAATAATAAAATTGCGGGTGTCAATAACAGAACTGAATACGTTGAA
 S  V  F  L  K  N  N  K  I  A  G  V  N  N  R  T  E  Y  V  E

                                                             1620
ACAACATCTACCGAGTACACTAGTGGAAAAATTAACCTGTCTCATCAAGGCGCGTATGTT
 T  T  S  T  E  Y  T  S  G  K  I  N  L  S  H  Q  G  A  Y  V

                                                             1680
GCTCAATATGAAATCCTTTGGGATGAAATCAATTATGATGACAAAGGAAAAGAAGTGATT
 A  Q  Y  E  I  L  W  D  E  I  N  Y  D  D  K  G  K  E  V  I

                                                             1740
ACAAAACGACGTTGGGATAACAACTGGTATAGTAAGACATCACCATTTAGCACAGTTATC
 T  K  R  R  W  D  N  N  W  Y  S  K  T  S  P  F  S  T  V  I

                                                             1800
CCACTAGGAGCTAATTCACGAAATATACGTATCATGGCTAGAGAG GCC ACCGGCTTAGCT
 P  L  G  A  N  S  R  N  I  R  I  M  A  R  E  A  T  G  L  A
```

SLO.DNA

```
                                                          1860
TGGATGGTGGCGAAAAGTGATCGACGAAAGAGATGTGAAACTGTCTAAAGAAATCAAT
 W  E  W  W  R  K  V  I  D  E  R  D  V  K  L  S  K  E  I  N

                                                          1920
GTCAACATCTCAGGATCAACCCTGAGCCCATATGGTTCGATTACTTATAAGTAGGACTGG
 V  N  I  S  G  S  T  L  S  P  Y  G  S  I  T  Y  K

TTCAAGAGGTTC
```

CYTOLYTIC STREPTOLYSIN O MUTANTS AND USES

This invention relates to cytolytic streptolysin O (SLO) mutants and their uses in diagnostic anti-streptolysin O antibody tests (ASO tests). In particular the present invention relates to the identification, construction and production of cytolytic SLO derivatives that do not contain any cysteine (Cys) amino acids. This invention also relates to the use of these SLO derivatives in diagnostic tests based on specific binding properties, such as binding between antigen and antibody, and the inhibition of the cytolytic activities of SLO and cytolytic SLO derivatives by anti-Streptolysin O antibodies in human serum.

SLO is a toxic cytolytic protein produced by *Streptococcus pyogenes* (*S.pyogenes*) which causes a number of human diseases. During infection, the gene encoding SLO is expressed and SLO is secreted by *S.pyogenes*. The SLO secreted by *S.pyogenes* is sensitive to oxidation and the cytolytic activity is greatly reduced after after a period of exposure to air. This lost activity is restored by the addition of reducing agents such as dithiothreitol (DDT). The sensitivity of SLO to oxidation is associated with the presence of a Cys amino acid in the native protein. All prior art indicates that this Cys amino acid is an essential amino acid for the cytolytic activity of SLO and this Cys amino acid is often referred to as the "essential Cys".

The infected human host produces anti-SLO antibodies to antigenic sites on the SLO molecule.Thus diagnostic tests detecting these anti-SLO antibodies in human serum, can indicate (past or present) infection by *S.pyogenes*.

The immunodiagnostic assays presently being used for detection of anti-SLO antibodies in human serum utilise active SLO protein prepared from cultures of *S.pyogenes*. These assays generally comprise the following steps:

(a) Take serum sample from patient.
(b) Make serial dilutions of serum sample in a suitable buffer.
(c) For each test include a control containing buffer but no serum.
(d) Add a standard quantity of active SLO to each dilution of serum and to the control.
(e) Incubate the mixtures for a standard time and at a standard temperature, to allow any anti-SLO antibodies in the mixtures to combine with, and neutralise the added SLO.
(f) Add a standard quantity of red blood cells to each mixture.
(g) Incubate the mixtures for a standard time and at a standard temperature to allow any active (non-neutralised) SLO to lyse the added cells.
(h) Determine the highest dilution of serum that has neutralised the added SLO, that is which corresponds to the dilution producing less than 50% lysis of the added red blood cells.

Thus, where the serum sample contains high levels of anti-SLO antibody, there will be neutralisation of the active SLO and therefore no lysis of the red blood cells. Conversely, where the serum sample does not contain antibodies to SLO, there will be no neutralisation and therefore there will be extensive lysis of the

red blood cells.

There are a number of problems with preparing active SLO from *S.pyogenes* cultures for use in these assays. These problems are caused by the sensitivity of SLO to degradation by proteases that are also secreted by *S.pyogenes* and by the sensitivity of SLO to oxidation due to the presence of a Cys amino acid in the molecule. The gene encoding SLO has been cloned and expressed in *Escherichia coli* (*E.coli*) and the complete nucleotide sequence of the cloned SLO gene has been determined and the amino acid sequence of SLO has been deduced from the nucleotide sequence of the gene. These data have been published (Kehoe and Timmis, 1984, Infection and Immunity 43:804-810 and Kehoe *et al* 1987, Infection and immunity 55:3228-3232). These data showed that the SLO protein contains only one Cys amino acid. The native SLO expressed by the cloned gene in *E.coli* could be used in the current immunodiagnostic assays. However the cloned gene product also contains the Cys amino acid. Prior art indicates that deletion of this single Cys amino acid or the exchange of this Cys amino acid for any other natural amino acid would irreversibly inactivate the cytolytic activity of SLO.

The problem addressed by the present application was that of producing a cytolytic derivative of SLO did not contain a Cys amino acid and that was not sensitive to oxidation. It was not at all clear that this could be done.

In one aspect the present invention provides data that shows that Cys is not an essential amino acid for the cytolytic activity of SLO.

In another aspect the present invention provides a DNA sequence encoding a cytolytic SLO derivative that does not contain a Cys amino acid.

In another aspect the present invention provides a cytolytic SLO derivative that does not contain a Cys amino acid.

In another aspect the present invention provides a process which comprises producing cytolytic SLO derivatives that do not contain a Cys amino acid by culturing a transformed cell so as to express the protein and recovering the protein therefrom.

The present invention also provides methods and diagnostic kits for detecting the presence or absence of antibodies to SLO in clinical samples and for quantifying the levels of such antibodies in these samples. A typical diagnostic method may comprise replacing native SLO in the current ASO assays (described above) with the cytolytic SLO derivatives that do not contain a Cys amino acid.

In order that the present invention may be more readily understood, embodiments will now be described with reference to Figure 1 which shows how the genes encoding cytolytic SLO derivatives (referred to as "MUTANT" in Fig. 1) were constructed and with reference to

Figure 2 which shows the complete nucleotide sequences of the cloned SLO gene and a mutant gene encoding a cytolytic SLO derivative that does not contain a Cys amino acid.

Figure 1 outlined the steps involved in constructing mutant SLO genes encoding SLO derivatives that do not contain a Cys amino acid. Plasmid pMK206 DNA was cleaved with the restriction enzymes SnaBI (S) and BamHI (B) and the two resulting DNA fragments were separated by electrophoresis on agarose gels and purified (highlighted by boxes). The smaller of these two purified DNA fragments was then cleaved with the restriction enzyme HpaII (Hp) and the two resulting fragments were separated. The larger Hp-B fragment was purified and the smaller S-Hp fragment was discarded. A double stranded oligonucleotide was prepared by DNA synthesis *in vitro* and hybridisation, such that it replaced the discarded segment of the SLO gene with a new sequence that did not contain a Cys amino acid codon, but that replaced this Cys codon with a codon for an alanine or some other amino acid. The purified large B-S fragment from the first restriction enzyme cleavage, the purified Hp-B fragment from the second restriction enzyme cleavage and the synthetic double stranded oligonucleotides were ligated together to produec new plasmids. These new recombinant plasmids were transformed into an *E.coli* host and progeny expressing cytolytic SLO derivatives that do not contain a Cys amino acid were identified. The sequence of one such derivative, where the Cys codon is replaced by a codon for the amino acid alanine is described in Figure 2. A procedure for producing the cytolytic SLO derivatives from the transformed *E.coli* cells was developed such that protein that could be used in immunodiagnostic tests could be provided.

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CLAIMS

1. A cytolytic derivative of the thiol-activated protein streptolysin O (SLO) comprising amino acid sequences with no cysteine (Cys) amino acid.

2. An SLO derivative according to claim 1 wherein SLO has been subjected to derivatisation at the Cys amino acid.

3. An SLO derivative according to claim 2 wherein the derivatisation involves altering the amino acid sequence by means amino acid substitution, insertion or deletion acheived by genetic manipulation of an SLO gene or any other means.

4. An SLO derivative according to claim 1 wherein the cytolytic activity is resistant to inactivation by oxidation or chemical modification of a thiol group.

5. A process which comprises the production of an SLO derivative of claim 3 by expression of the protein in a recombinant host cell from DNA encoding the derivative.

6. A diagnostic kit for detecting the presence or absence of antibodies to SLO in a clinical sample, which comprises an SLO derivative of claim 1 together with ancillary components for measuring cytolytic activity.

7. A method for detecting the presence or absence of antibodies to SLO in a clinical sample, which method comprises contacting dilutions of the sample with an SLO derivative of claim 1 and measuring the cytolytic activity remaining after contact between the sample dilutions and the SLO derivative of claim 1.

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